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Exhibit 1

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Sonic hedgehog and *Fgf-4* Act through a Signaling Cascade and Feedback Loop To Integrate Growth and Patterning of the Developing Limb Bud

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Summary

Proper limb growth and patterning requires signals from the zone of polarizing activity in the posterior mesoderm and from the overlying apical ectodermal ridge (AER). *Sonic hedgehog* and *Fgf-4*, respectively, have recently been identified as candidates for these signals. We have dissected the roles of these secreted proteins in early limb development by ectopically regulating their activities in a number of surgical contexts. Our results indicate that *Sonic hedgehog* initiates expression of secondary signaling molecules, including *Bmp-2* in the mesoderm and *Fgf-4* in the ectoderm. The mesoderm requires ectodermally derived competence factors, which include *Fgf-4*, to activate target gene expression in response to *Sonic hedgehog*. The expression of *Sonic hedgehog* and *Fgf-4* is coordinately regulated by a positive feedback loop operating between the posterior mesoderm and the overlying AER. Taken together, these data provide a basis for understanding the integration of growth and patterning in the developing limb.

Introduction

Sonic hedgehog encodes a key inductive signal used in patterning several different embryonic structures, including the neural tube, the somites, and the limb bud (Echelard et al., 1993; Krauss et al., 1993; Riddle et al., 1993; Roelink et al., 1994). In the limb bud, *Sonic hedgehog* appears to mediate the function of the zone of polarizing activity (ZPA), a signaling center essential for limb patterning. The ZPA is operationally defined as a region of posterior limb bud mesoderm that, when transplanted underneath the apical ectodermal ridge (AER) on the anterior margin of another limb bud, induces symmetrical duplications of the normal limb elements reflected about the anterior–posterior (AP) axis (a property called polarizing activity) (Saunders and Gasseling, 1968; Tabin, 1991; Tickle, 1991).

Several genes that might normally be regulated by *Sonic hedgehog* during limb development have been identified. The *Hoxd-9* through *Hoxd-13* genes are transcription factors expressed in a nested pattern initiating along the posterior margin of the developing limb bud, and they are involved in regulating the pattern of chondrogenic condensations in the limb (Izpisúa-Belmonte et al., 1991; Yokouchi et al., 1991; Morgan et al., 1992; Dollé et al., 1993; Davis and Capecchi, 1994). *Hoxd-11*, *-12*, and *-13* are known to be downstream of the ZPA because their expression is induced ectopically by ZPA grafts (Nohno et al., 1991; Koyama et al., 1993). Similarly, ectopic expression of *Sonic hedgehog* in the anterior limb bud is known to induce expression of *Hoxd-11* and *Hoxd-13* (Riddle et al., 1993). It is not known whether the endogenous expression of these genes is induced by *Sonic hedgehog*. Another gene that is downstream of the ZPA is *Bmp-2* (Francis et al., 1994). This transforming growth factor β family member is normally expressed in the posterior mesoderm and is also ectopically induced by ZPA grafts. Since it is a secreted factor, *Bmp-2* could function in limb patterning as a secondary signal elicited in response to *Sonic hedgehog*, although at present its role in this regard is unresolved (Niswander and Martin, 1993; Francis et al., 1994).

While it has been generally assumed that the ZPA, and by extension *Sonic hedgehog*, acts directly on the mesoderm to specify identity along the AP axis, the actual target cells of Sonic hedgehog protein have not been defined. Sonic hedgehog might act directly on the mesoderm, indirectly through the ectoderm, or through a combination of the two to specify mesodermal positional identities. Similarly, it is not clear whether *Sonic hedgehog* regulates gene expression only in those cells that bind Sonic hedgehog protein, or whether it acts indirectly through a cascade of secondary signaling molecules.

Because of its dramatic effect on AP polarity, the ZPA has primarily been considered as a regulator of AP pattern. However, the ectopic outgrowth induced by a ZPA graft is also appropriately patterned along its proximal–distal (PD) axis. The control of outgrowth and PD patterning by the ZPA is generally considered to be an indirect effect on the mesoderm, mediated through the AER, the other clearly defined signaling center in the limb.

The morphologically distinct AER extends anteroposteriorly along the distal margin of the bud. It has several functions, including regulating limb outgrowth and PD pattern. The AER sends mitogenic signals to the immediately subjacent mesodermal tissue, the progress zone, thereby inducing distal outgrowth of the limb bud. As limb outgrowth proceeds, the cells leaving the progress zone are specified to progressively more distal fates, resulting in establishment of the PD axis. The AER is additionally required for the maintenance of a functional ZPA (Todt and Fallon, 1987; Niswander et al., 1993; Vogel and Tickle, 1993).

Most of the properties of the AER seem to be conveyed by members of the fibroblast growth factor (FGF) family (reviewed by Laufer, 1993; Niswander et al., 1993; Vogel and Tickle, 1993; Fallon et al., 1994). *Fgf-2* and *Fgf-4* are expressed in the AER, and their proteins can functionally replace the AER to promote limb outgrowth and patterning as well as to maintain the ZPA (Niswander and Martin, 1992; Savage et al., 1993; Fallon et al., 1994). However,

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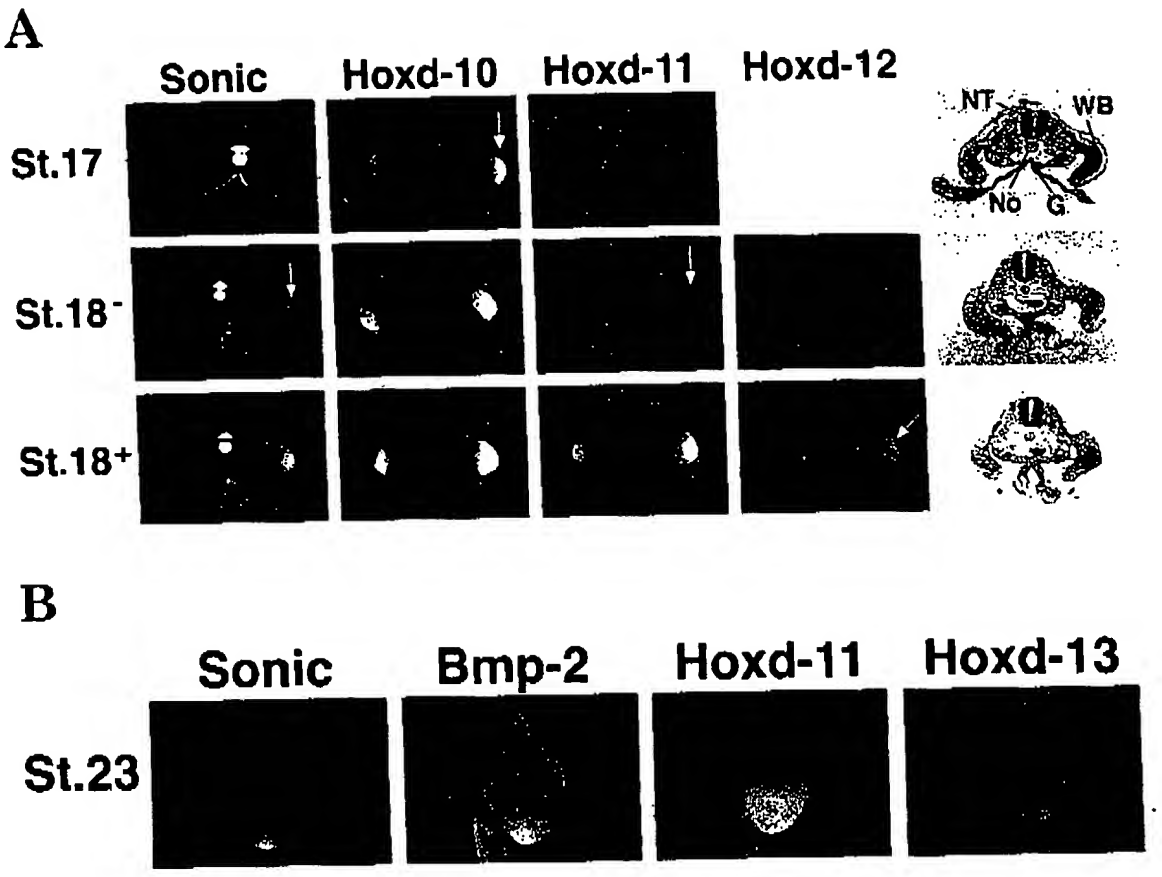

Figure 1. Spatial and Temporal Comparison of *Sonic hedgehog*, *Hoxd*, and *Bmp-2* Gene Expression

Serial sections of individual embryos of a staged series were hybridized with radioactive probes for *Sonic hedgehog*, *Hoxd-10*, *Hoxd-11*, and *Hoxd-12*. Several embryos were analyzed at each stage with an effective maximum of 35 μm separating any two sections analyzed with a given probe. Thus, within the limits of probe sensitivity, it is unlikely that any expression domains have gone undetected.

(A) Transverse sections at the level of the posterior wing bud showing the relative onset of expression of these four genes. The first detectable signal of each gene is indicated by an open arrow. *Hoxd-10* is expressed prior to *Sonic hedgehog*, and *Sonic hedgehog* and *Hoxd-11* expression initiate coincidently and are followed by *Hoxd-12*. Only the right wing bud in each section should be used for comparison as sections were cut obliquely. Embryo stages (St.) and probes used are indicated in the left and top margins, and panels to the right are bright field images. Abbreviations: WB, wing bud; No, notochord; NT, neural tube; and G, gut endoderm.

(B) Serial sections of a stage 23 wing bud hybridized with *Sonic hedgehog*, *Bmp-2*, *Hoxd-11*, and *Hoxd-13* probes. Note that the *Sonic hedgehog* and *Bmp-2* expression domains are both centered at the posterior wing margin. *Hoxd-11* expression extends throughout the PD extent of the bud and anteriorly to mid-bud level, while the *Hoxd-13* expression domain has shifted distally. Anterior is to the top; distal is to the right.

it is not clear which, if either, of these genes normally functions in these capacities.

The ZPA region of the limb mesoderm is required during normal limb development to maintain and polarize the AER (reviewed by Tabin, 1991; Tickle, 1991). Thus, there appears to be a signaling pathway from the ZPA to the AER in addition to the pathway from the AER that maintains the ZPA. The reciprocal dependence between the AER and the ZPA makes it difficult to dissect the mechanisms by which these signaling centers organize pattern. The ability to replace the ZPA with a source of *Sonic hedgehog* that is not dependent on the AER or to replace the AER with a source of FGF that is not dependent on the ZPA allows us to manipulate the activity of the two signaling centers independently. In this way, the mechanisms by which each acts and the interaction of these centers to generate pattern can be separately addressed at a molecular level. Here, we examine the interrelationship between *Sonic hedgehog* and *Fgf-4* and their potential targets, *Bmp-2* and the *Hoxd* genes, in the limb buds of normal and manipulated embryos to elucidate their roles in the regulation of limb pattern.

We find that *Sonic hedgehog* generates secondary signals in both the mesoderm (*Bmp-2*) and ectoderm (*Fgf-4*). Moreover, *Fgf-4* produced by the AER affects ZPA function in two ways: it induces competence within the mesoderm required for gene induction by *Sonic hedgehog*, and it functions as part of a positive feedback loop that maintains *Sonic hedgehog* expression. The use of a common signal, FGF, to induce proliferation, promote competence to re-

spond to *Sonic hedgehog*, and to maintain *Sonic hedgehog* expression results in the coordination of patterning and outgrowth in the limb.

Results

Relationship of *Sonic hedgehog* to Endogenous *Bmp-2* and *Hoxd* Gene Expression

The best candidates for genes regulated by *Sonic hedgehog* in vivo are the distal members of the *Hoxd* gene cluster, *Hoxd-9* through *Hoxd-13*, and *Bmp-2*. We analyzed the relative expression domains of these genes in chick limb buds. *Hoxd-9* and *Hoxd-10* are expressed throughout the presumptive wing field at stage 16 (Hamburger and Hamilton, 1951), prior to the first detectable expression of *Sonic hedgehog* at early stage 18 (Figure 1A). *Hoxd-11* expression is first detectable at early stage 18, the same time as is *Sonic hedgehog*, in a domain coextensive with *Sonic hedgehog*. Expression of *Hoxd-12* and *Hoxd-13* commences shortly thereafter. These results suggest that *Sonic hedgehog* normally induces, directly or indirectly, the expression of only the latter three members of the cluster even though all five are nested within the early limb bud.

As limb outgrowth proceeds, *Sonic hedgehog* expression remains at the posterior margin of the bud. In contrast, the *Hoxd* gene expression domains are very dynamic and lose their concentric nesting around the *Sonic hedgehog* expression domain. By stage 23, the *Hoxd-11* domain extends anteriorly and distally far beyond that of *Sonic hedgehog*, while *Hoxd-13* expression becomes biased distally but overlaps *Sonic hedgehog* (Figure 1B; Figure 2).

While it is not clear whether *Bmp-2* is expressed before *Sonic hedgehog* (see Francis et al., 1994), *Bmp-2* is expressed in a mesodermal domain that apparently overlaps and surrounds that of *Sonic hedgehog* at the earliest stages of *Sonic hedgehog* expression. As the limb bud develops, the mesodermal expression of *Bmp-2* remains near the posterior limb margin and is centered around that of *Sonic hedgehog*, but in a domain larger than that of *Sonic hedgehog* (see Figure 1B; data not shown). This correspondence between *Sonic hedgehog* and *Bmp-2* expression lasts until around stage 25, much longer than does the correspondence between *Sonic hedgehog* and *Hoxd* gene expression.

Relationship of *Sonic hedgehog* to Induced *Bmp-2* and *Hoxd* Gene Expression

The fact that the expression domains of the *Hoxd* genes diverge over time from that of *Sonic hedgehog* implies that *Sonic hedgehog* does not directly regulate their later patterns of expression. However, the later expression domains could be genetically downstream of *Sonic hedgehog*. If this were the case, the program of *Hoxd* gene expression initiated by exogenous *Sonic hedgehog* should recapitulate that seen endogenously. To test this, the anterior marginal mesoderm of early bud (stages 18–20) wings was injected at a single point under the AER with a replication-competent virus that expresses a chicken *Sonic hedgehog* cDNA. The *Sonic hedgehog* and *Hoxd* gene expression domains in the infected limbs were analyzed in

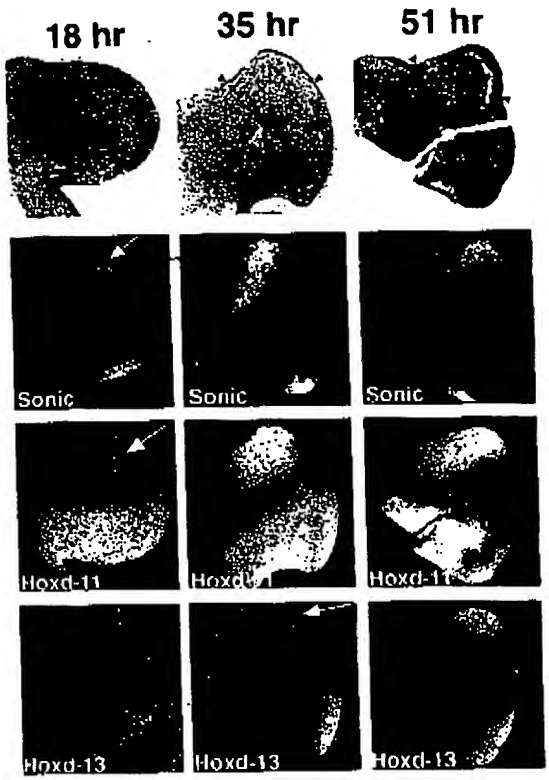

Figure 2. Time Course of *Hoxd* Gene Induction Following *Sonic hedgehog* Virus Injection

Embryos injected with *Sonic hedgehog* virus at the anterior wing margin were harvested at different times after infection, sectioned serially, and hybridized with probes for *Sonic hedgehog*, *Hoxd-11*, and *Hoxd-13*. In each instance, the endogenous expression domains are visible in the posterior half of the limb bud. Viral message and induced *Hoxd-11* expression are first detected in closely overlapping domains at 18 hr (open arrows). *Hoxd-13* expression is detected at 35 hr, by which time the virus has spread distally and laterally within the ectopic growth (closed arrowheads on bright field sections), and the *Hoxd-11* domain has expanded distally and anteroproximally such that it resembles the endogenous domain. By 51 hr after infection, the *Hoxd-13* domain is spreading distally but occupies only a small fraction of the total infected area. Anterior is to the top; distal is to the right.

sectioned and intact embryos (Figure 2; data not shown). Viral *Sonic hedgehog* message is first detected approximately 18 hr after infection at the anterior margin at the same time as, and approximately coextensively with, induced *Hoxd-11*. This suggests that *Sonic hedgehog* can rapidly induce *Hoxd-11* expression and that the lag after injection represents the time required to achieve *Sonic hedgehog* expression. By 35 hr post infection, *Hoxd-11* expression has expanded both anteroproximally and distally, with respect to the wedge of *Sonic hedgehog*–expressing cells, into a domain that appears to mirror the more distal aspects of the endogenous *Hoxd-11* domain. Weak *Hoxd-13* expression is also detected at 35 hr at the distal margin of the *Sonic hedgehog*–expressing domain. At 51 hr after infection, the relationship of *Sonic hedgehog* and *Hoxd-11* expression is similar to that seen at 35 hr, while the induced *Hoxd-13* expression has reached wild-type

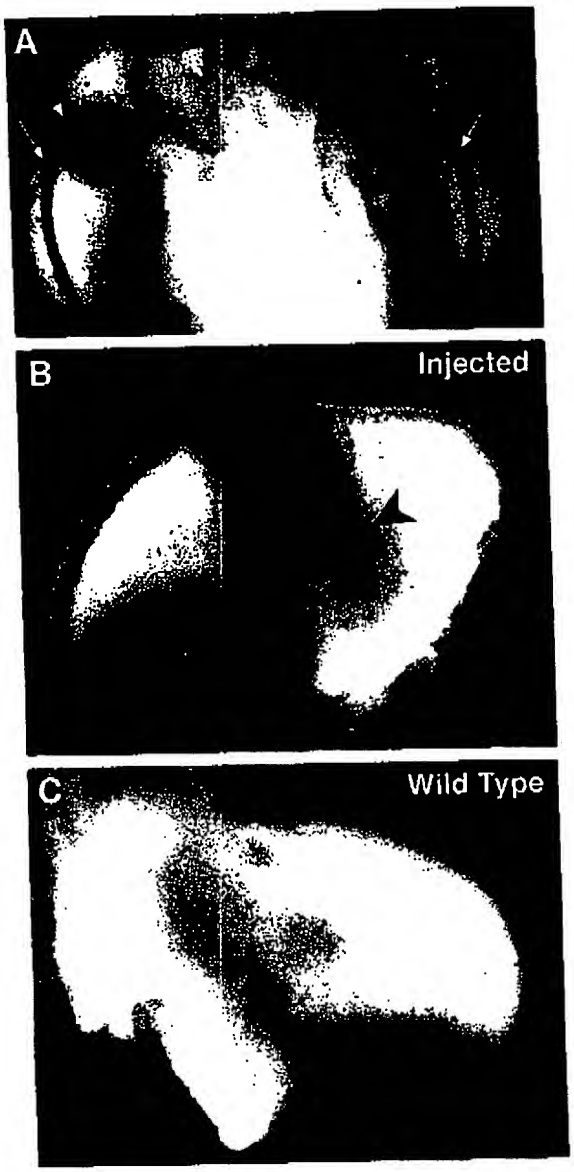

Figure 3. *Sonic hedgehog* Induces *Bmp-2* Expression in the Limb Mesoderm and AER

A stage 18 chick embryo injected with *Sonic hedgehog* virus in the right anterior wing bud mesoderm was harvested 30 hr after infection. *Bmp-2* and *Sonic hedgehog* message were detected by two-color whole-mount in situ hybridization. *Bmp-2* expression is shown in purple; the *Sonic hedgehog* message is shown in magenta.

(A) Anterior view of the wing buds (ventral view of the embryo) following detection of *Bmp-2* alone. Induced mesodermal expression of *Bmp-2* in the injected wing is indicated by the closed arrowhead. *Bmp-2* expression in the AER is visible as a stripe running towards the distal tip of the bud. The normal extent of the AER is indicated by the open arrows; expression in the proximally induced AER extends to the open arrowhead.

(B and C) Ventral views of the injected and control wings of embryo in (A) stained for *Sonic hedgehog* and *Bmp-2* message. The viral message (red arrowhead) is detected in a slightly broader domain than the induced mesodermal *Bmp-2* expression domain. Note the endogenous *Bmp-2* expression domain in the posterior of the limb bud and along the entire AER.

levels and is restricted to the distal portions of the ectopic growth. Thus, the ectopic *Hoxd* expression domains better reflect their endogenous patterns of expression than they reflect the *Sonic hedgehog* expression domain. This suggests that there are multiple factors regulating *Hoxd* expression and that the actions of these factors lie downstream of *Sonic hedgehog*.

Bmp-2 is normally expressed in two places in the early limb bud, in the posterior mesoderm and throughout the AER (Francis et al., 1994). In injected limb buds, additional *Bmp-2* expression is seen in both the anterior mesoderm and in the anteriorly extended AER. The domain of *Bmp-2* expression is slightly more restricted than that of viral expression, suggesting a delay in *Bmp-2* induction (Figure 3; data not shown). *Bmp-2* expression in both the mesoderm and ectoderm is thus a downstream target of *Sonic hedgehog* activity in the mesoderm. In contrast with the expression domains of the *Hoxd* genes, the endogenous and ectopic *Bmp-2* expression domains correlate well with those of *Sonic hedgehog*. This suggests that *Bmp-2* expression is regulated more directly by *Sonic hedgehog* than is expression of the *Hoxd* genes.

The AER and Competence to Respond to *Sonic hedgehog*

Ectopic activation of *Hoxd* gene expression is biased distally in virally infected regions, suggesting that ectodermal factors, possibly from the AER, are required for *Hoxd* gene induction by *Sonic hedgehog*. To test this, *Sonic hedgehog* virus was injected into the proximal–medial mesoderm of stage 21 limb buds, presumably beyond the influence of the AER. Although the level of *Sonic hedgehog* expression was comparable to that observed in distal injections, proximal misexpression of *Sonic hedgehog* did not result in ectopic induction of *Hoxd* genes or *Bmp-2*, nor did it result in any obvious morphological effect (data not shown). The lack of gene induction following proximal misexpression of *Sonic hedgehog* suggests that exposure to *Sonic hedgehog* alone is insufficient to induce expression of these genes.

This apparent requirement for the AER was tested more rigorously by injection of *Sonic hedgehog* virus into the anterior marginal mesoderm of stage 20/21 limb buds after the anterior half of the AER had been surgically removed. Embryos were allowed to develop for a further 36–48 hr before harvesting. During this time, the AER remaining on the posterior half of the limb bud promotes almost wild-type outgrowth and patterning of the bud. Gene expression was monitored both in sectioned and intact embryos. In the presence of the AER, *Sonic hedgehog* induces both anterior mesodermal proliferation and expression of *Hoxd-11*, *Hoxd-13*, and *Bmp-2*. In the absence of the overlying AER, *Sonic hedgehog* does not induce either mesodermal proliferation or expression of these genes above background (Figure 4; data not shown). Signals from the AER are thus required to allow both the proliferative and patterning effects of *Sonic hedgehog* on the mesoderm.

Since application of FGF protein can rescue other functions of the AER, we sought to determine whether FGFs might promote mesodermal competence to respond to

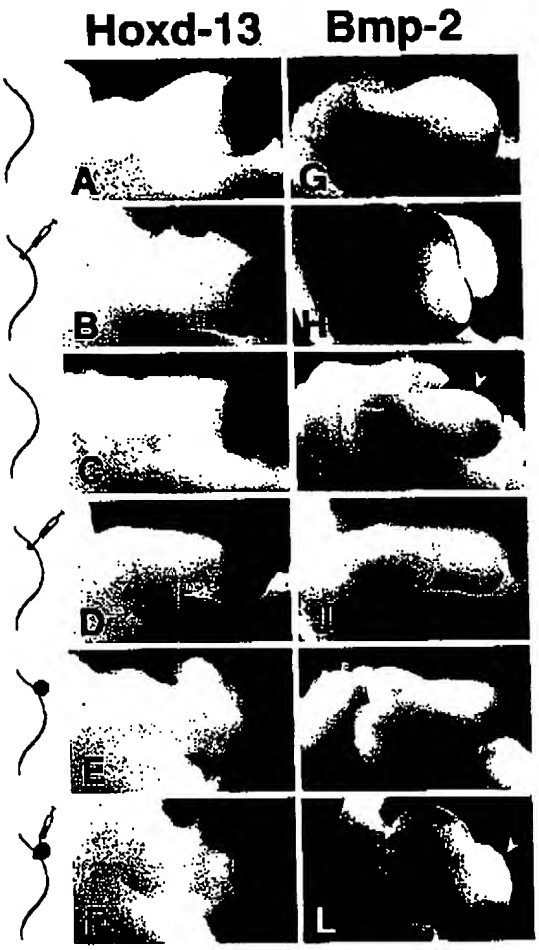

Figure 4. Mesodermal Induction of Gene Expression by *Sonic hedgehog* Requires the AER, Which Can Be Substituted by FGF-4 Protein

Embryos from which the anterior AER of the wing had been removed and injected with *Sonic hedgehog* virus either in the absence or presence of FGF-4-soaked beads were harvested after 36–41 hr. Individual protocols are indicated schematically to the left: the thickened line is the AER, the red syringe indicates *Sonic hedgehog* virus, and the blue circle represents an FGF-4-soaked bead. (A)–(F) were hybridized with a *Hoxd-13* probe, and (G)–(L) were hybridized with a *Bmp-2* probe.

(A and G) Unoperated limbs showing the normal domain of *Hoxd-13* (A) and *Bmp-2* (G) expression in a stage 25 limb bud. AER expression of *Bmp-2* is not visable in (G) owing to the angle at which the photograph was taken.

(B and H) This is a control *Sonic hedgehog* virus injection, and the AER is intact. Note anterior induction of mesodermal proliferation and mesodermal *Hoxd-13* and *Bmp-2* expression. Note also the induced AER that expresses *Bmp-2* in (H).

(C and I) Control AER removal. Neither *Hoxd-13* nor *Bmp-2* is significantly expressed in the anterior. AER expression of *Bmp-2* serves as a control for the completeness of AER removal (open arrowhead in [I]).

(D and J) There is *Sonic hedgehog* virus, and the AER is removed. Note the similarity with (C) and (I). There is no induction of *Hoxd-13* or *Bmp-2* gene expression above background and there is no induction of mesodermal proliferation.

(E and K) There is an FGF-4 bead, and the AER is removed. There is strong induction of anterior mesodermal proliferation, and there is no induction of *Hoxd-13* or *Bmp-2* expression above control levels.

(F and L) There is *Sonic hedgehog* virus and the FGF-4 bead, and the AER is removed. There is strong induction of mesodermal proliferation, which expresses both *Hoxd-13* and *Bmp-2*. Note the anterior extent of the remaining AER, which expresses *Bmp-2* (open arrowhead).

Note: In control embryos not infected with *Sonic hedgehog* virus, weak induction of *Hoxd-11* and *Bmp-2*, but not *Hoxd-13*, expression is observed in the anterior mesodermal border following removal of the AER. The expression levels are always significantly less than those of either the endogenous gene or that induced by *Sonic hedgehog* in the presence of a functional AER. In embryos in which the AER was replaced by an FGF-4 bead but was not infected with *Sonic hedgehog* virus, these weak anterior expression domains of *Hoxd-11* and *Bmp-2* are larger than those seen following the surgery alone.

Sonic hedgehog. FGF-4-soaked beads were stapled to AER-denuded anterior mesoderm that was infected with *Sonic hedgehog* virus. In the presence of both *Sonic hedgehog* virus and FGF-4 protein, *Hoxd-11*, *Hoxd-13*, and *Bmp-2* expression are all induced (Figure 4; data not shown). The expression levels and domains of the induced genes are similar to their endogenous expression and to their induction in the presence of the AER. Thus, *Fgf-4* can induce the competence of the mesoderm to respond to *Sonic hedgehog*.

Sonic hedgehog alone is insufficient to induce either gene expression or mesodermal proliferation in the absence of the AER. We next asked whether FGF-4 alone has any effect on gene induction or mesodermal proliferation. Application of FGF-4 in the absence of *Sonic hedgehog* virus does not induce *Hoxd* or *Bmp-2* gene expression above control levels; however, FGF-4 alone induces mesodermal outgrowth (Figure 4; data not shown). These results suggest that mesodermal gene activation requires direct action of Sonic hedgehog on the mesoderm and that the proliferation of the mesoderm in response to Sonic hedgehog is indirect, owing to the induction of FGFs.

Sonic hedgehog Induces Polarized *Fgf-4* Expression in the AER

Fgf4 is expressed in a graded fashion in the AER of the mouse limb bud, with maximal expression at the posterior region of the AER tapering to undetectable levels in the anterior ridge (Niswander and Martin, 1992). Therefore, we decided to investigate whether *Fgf-4* is asymmetrically expressed in the chick AER and whether its expression is induced by *Sonic hedgehog*. A fragment of the chicken *Fgf-4* gene was cloned from a stage 22 chicken limb library by polymerase chain reaction (PCR) using degenerate primers designed from mouse *Fgf4* and Xenopus *e-Fgf* sequences (based on information provided by L. Niswander and G. Martin). Assignment of gene identity was based on primary sequence as well as comparison of expression patterns with that of murine *Fgf4* (Niswander and Martin, 1992; see Figures 6 and 7; data not shown). Whole-mount in situ hybridization analysis showed strong limb expression of chick *Fgf-4* in the AER. *Fgf-4*, like *Bmp-2*, is expressed in the posterior end of the AER but stops anteriorly before the morphological end of the AER, which was also observed by Niswander et al. (1994). Expression is first detected in the distal AER at about stage 18. As

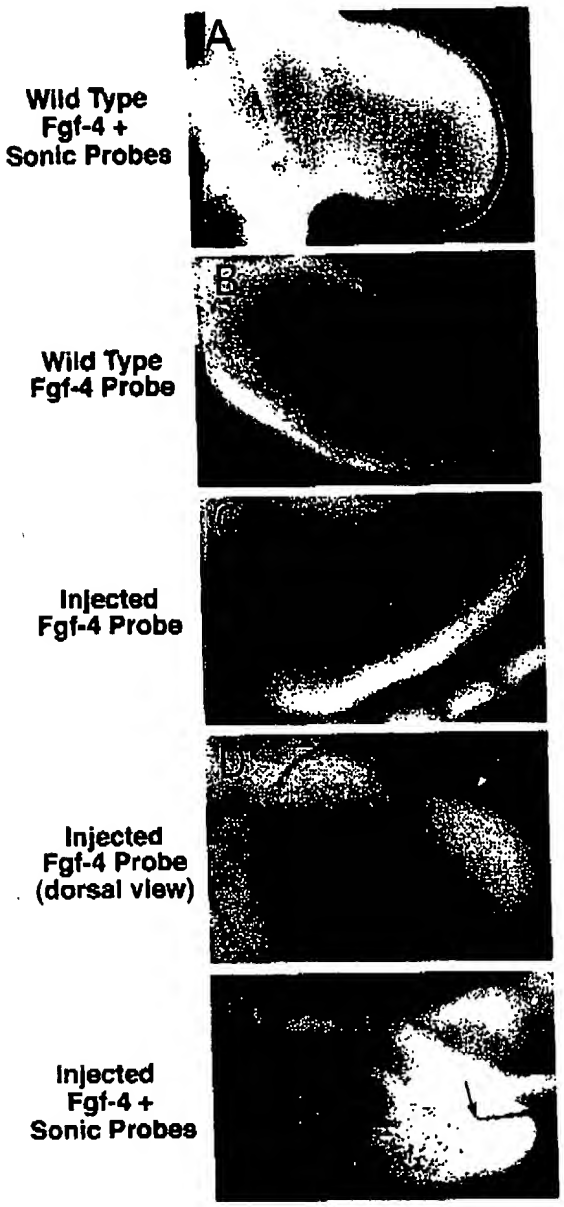

Figure 5. *Sonic hedgehog* Induces *Fgf-4* in the AER

(A) Stage 24 limb bud that was hybridized with probes for both *Sonic hedgehog* (magenta) and *Fgf-4* (purple) expression. *Sonic hedgehog* is expressed in the posterior distal mesoderm, and *Fgf-4* is expressed in the AER, biased towards the posterior.

(B–E) Stage 18/19 chick embryos injected with *Sonic hedgehog* virus in the right anterior wing bud mesoderm, harvested 36 hr after infection, and hybridized for *Fgf-4* (purple) expression (B, C, D) or for both *Fgf-4* and *Sonic hedgehog* (magenta) expression (E). (B) Anterior view of uninfected left wing. *Fgf-4* expression within AER (closed arrow) does not extend as far to anterior as the morphological AER does (closed arrowhead). The photo has been flipped for ease of comparison with the right wing (C). (C and D) Infected embryo showing induced, polarized *Fgf-4* expression in ectopic AER. Anterior (C) and dorsal (D) view of right, injected wing of embryo in (B). Ectopic *Fgf-4* expression is seen on the proximal side of induced AER (blue arrowhead). Anterior extent of endogenous *Fgf-4* expression is indicated by the closed (C) or open (D) arrows. (E) Infected embryo showing induced *Fgf-4* expression and the extent of viral infection. Ectopic *Fgf-4* is visible at the distal margin (blue arrowhead) of the ectoderm overlying the viral infection (magenta stain). The anterior extent of the endogenous *Fgf-4* expression within the AER is also indicated (closed arrow).

Under hybridization conditions that optimize detection of expression in the ectoderm, additional *Fgf-4* signal is visible in non-AER ectoderm as large flecks of staining lateral and proximal to the ends of the AER expression domains (B, C, D, E). This is not visible under hybridization conditions that optimize detection of expression in the mesoderm (A). Weak expression is also detected in the anterior marginal mesoderm at ankle level in stage 25/26 legs (data not shown).

In (A) and (D), proximal is to the left, and anterior is to the top; in (B), (C), and (E), proximal is to the left, and ventral is to the top.

outgrowth proceeds, the posterior bias develops. Expression peaks around stage 24/25 and fades by stage 28/29.

The expression domain of *Fgf-4* becomes posteriorly biased as *Sonic hedgehog* is expressed in the posterior mesoderm. This observation is consistent with *Sonic hedgehog* influencing the expression of *Fgf-4* in the posterior AER (Figure 5A). To test the effect of *Sonic hedgehog* on *Fgf-4* expression in the AER, stage 18–20 embryos were infected with *Sonic hedgehog* virus in a single point at their anterior margin beyond the anterior limit of the AER. The embryos were harvested 1–2 days later, when an extension of the anterior AER became apparent. The expression of *Fgf-4* was analyzed by in situ hybridization (Figures 5B–5E). *Fgf-4* expression is induced in the anteriormost segment of the AER, in a region that is discontinuous with the endogenous expression domain and that overlies the domain of viral *Sonic hedgehog* infection. This result contrasts with the *Bmp-2* expression induced in the extended AER, which is always continuous with the endogenous expression domain (see Figure 3; see Figure 4H). The asymmetry of the induced *Fgf-4* expression indicates that *Sonic hedgehog* polarizes the extended AER, much as a ZPA graft does (Maccabe and Parker, 1979). Since FGFs by themselves are mitogenic for limb mesoderm, these results suggest *Sonic hedgehog* induces distal proliferation indirectly, through the induction of *Fgf* in the overlying AER.

Reciprocal Regulation of *Sonic hedgehog* by *Fgf-4*

Sonic hedgehog thus appears to be upstream of *Fgf-4* expression in the AER. However, since the AER is required to maintain polarizing activity in the posterior mesoderm (Vogel and Tickle, 1993; Niswander et al., 1993), *Sonic hedgehog* may also be downstream of the AER. If *Sonic hedgehog* is regulated by the AER and if the AER is regulated by *Sonic hedgehog*, this would imply that they are reinforcing one another through a positive feedback loop.

To test whether the AER dependence of ZPA activity is controlled at the level of transcription of the *Sonic hedgehog* gene, we assayed *Sonic hedgehog* expression following removal of the AER from the posterior half of the limb bud (Figure 6). *Sonic hedgehog* expression is reduced in an operated limb compared with the contralateral control limb within 10 hr of AER removal, indicating that *Sonic hedgehog* expression is indeed AER dependent. The dependence of *Sonic hedgehog* expression on signals from the AER suggests that one of the functions of the AER is

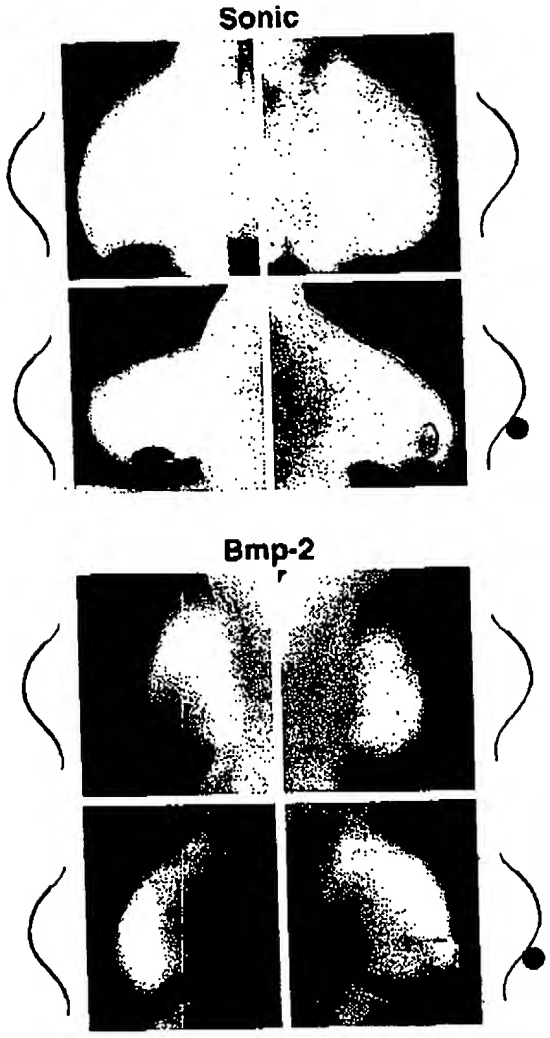

Figure 6. Mesodermal *Sonic hedgehog* and *Bmp-2* Expression Are Dependent on the AER and Can Be Rescued by FGF-4 Protein

The posterior half AER of stage 20/21 wing buds was either removed or replaced with an FGF-4-soaked bead, as indicated by the diagrams in which the AER is represented by the thick line and FGF-4 bead is represented by the blue circle. The embryos without beads were harvested after 10 hr, and embryos with beads were harvested after 24 hr. Contralateral limbs of embryos hybridized with either *Sonic hedgehog* or *Bmp-2* probes are shown horizontally, in pairs. In each case, *Sonic hedgehog* and *Bmp-2* expression are rapidly lost compared with the control bud, while the FGF-4 application rescues expression to approximately the same intensity and location as the control. Anterior is to the top, and proximal is towards the middle of each set of panels.

to constrain *Sonic hedgehog* expression to the more distal regions of the posterior mesoderm.

In addition to their mitogenic and competence-inducing properties, FGFs can also substitute for the AER to maintain the ZPA. In order to test whether FGFs can support the expression of *Sonic hedgehog*, beads soaked in FGF-4 protein were stapled to the posterior–distal tips of limb buds after posterior AER removal. Embryos were assayed for *Sonic hedgehog* expression approximately 24 hr later, when *Sonic hedgehog* expression is greatly reduced in operated limb buds that had not received an FGF-4 bead (Figure 6). Strong *Sonic hedgehog* expression is detectable in the posterior mesoderm, slightly proximal to the bead implant, and in a pattern reflecting the normal domain of *Sonic hedgehog* expression seen in the contralateral limb. With the finding that FGF-4 can maintain *Sonic hedgehog* expression, the elements required for a positive feedback loop between *Sonic hedgehog* expression in the posterior mesoderm and *Fgf-4* expression in the posterior AER are established (see also Niswander et al., 1994).

The domain of *Bmp-2* expression correlates well over time with that of *Sonic hedgehog*. We were therefore interested to learn whether the continued expression of *Bmp-2* requires signals from the AER, and if so, whether they could be replaced by FGF-4. To test this, we assayed *Bmp-2* expression following posterior AER removal and following its substitution with an FGF-4 bead (Figure 6). *Bmp-2* expression fades within hours of AER removal and can be rescued by FGF-4. These data indicate that the maintenance of *Bmp-2* expression in the posterior mesoderm, like that of *Sonic hedgehog*, is dependent on signals from the AER, which are likely to be FGFs.

Discussion

Patterning and outgrowth of the developing limb are known to be regulated by two major signaling centers, the ZPA and AER. The identification of *Sonic hedgehog* and FGFs as molecular mediators of the activities of the ZPA and AER has allowed us to dissociate the activities of these signaling centers from their regulation and to investigate the signaling pathways through which they function.

Our results suggest that the ability of cells to respond to Sonic hedgehog protein is dependent on FGFs produced by the AER. We also find that *Sonic hedgehog* induces a cascade of secondary signals involved in regulating mesodermal gene expression patterns. In addition, we find evidence for a positive feedback loop initiated by *Sonic hedgehog*, which maintains expression of *Sonic hedgehog* in the posterior mesoderm and *Fgf-4* in the AER. The feedback loop described suggests a mechanism by which outgrowth and patterning along the AP and PD axes of the limb can be coordinately regulated.

The Mesodermal Response to *Sonic hedgehog*

We have found that only mesoderm underlying the AER is responsive to *Sonic hedgehog* because the AER is required to provide competence signals to the limb mesoderm. *Fgf-4*, which is expressed in the AER, can substitute for the AER in this regard and, thus, acts in combination with *Sonic hedgehog* to promote *Hoxd* and *Bmp-2* gene expression in the mesoderm. FGFs may be permissive factors in a number of instructive pathways as they are also required for activins to pattern Xenopus axial mesoderm (Cornell and Kimelman, 1994; LaBonne and Whitman, 1994).

The induction of *Hoxd* and *Bmp-2* expression in response to *Sonic hedgehog* and FGF-4 in the absence of

an AER suggests that the mesoderm is a direct target tissue of Sonic hedgehog protein. Since *Sonic hedgehog* can induce *Fgf-4* expression in the AER, it follows that *Sonic hedgehog* also acts indirectly on the mesoderm through the induction of competence factors in the AER.

Downstream Targets and a Cascade of Signals Induced by *Sonic hedgehog*

The five *Abdominal-B*-like *Hoxd* genes, *Hoxd-9* through *Hoxd-13*, are initially expressed in a nested pattern centered on the posterior of the limb bud, a pattern that suggests they might be controlled by a common mechanism (Dollé et al., 1989; Izpisúa-Belmonte et al., 1991). Our analysis of the endogenous and induced domains of *Hoxd* gene expression suggests that *Sonic hedgehog* normally induces expression of *Hoxd-11*, *-12*, and *-13*. In contrast, we find that *Hoxd-9* an *-10* expression initiate before *Sonic hedgehog* mRNA is detectable. This implies that at least two distinct mechanisms control the initiation of *Hoxd* gene expression in the wing bud, only one of which is dependent on *Sonic hedgehog*.

Several observations suggest that the elaboration of the *Hoxd* expression domains is not controlled directly by *Sonic hedgehog* but rather by signals that are downstream of *Sonic hedgehog*. The *Hoxd* expression domains rapidly diverge from *Sonic hedgehog* and evolve into several distinct subdomains (C. E. N, B. A. M, A. C. Burke, E. L., E. DiMambro, and C. T., unpublished data). Moreover, these subdomains appear to be separately regulated, as analysis of the murine *Hoxd11* gene promoter suggests that it contains independent posterior and distal elements (Gérard et al., 1993). In addition, although initiation of *Hoxd-11* through *-13* gene expression is dependent on the AER, their expression is maintained following AER removal (Izpisúa-Belmonte et al., 1992). As *Sonic hedgehog* expression fades rapidly under similar conditions, this implies that maintenance of *Hoxd* gene expression is independent of *Sonic hedgehog*. Since ectopic *Sonic hedgehog* can induce a recapitulation of the *Hoxd* expression domains in the limb, we conclude that although indirect effectors appear to regulate the proper patterning of the *Hoxd* expression domains, they are downstream of *Sonic hedgehog*. Potential mediators of these indirect effects include *Bmp-2* in the mesoderm and *Fgf-4* from the AER.

In contrast with that of the *Hoxd* genes, *Bmp-2* gene expression in the posterior limb mesoderm appears to be continually regulated by *Sonic hedgehog*. We found that both endogenous and ectopic *Bmp-2* expression domains correspond to those of *Sonic hedgehog*. Furthermore, continued *Bmp-2* expression is dependent on the AER and can be rescued by FGF-4. It is likely that this is an indirect consequence of the fact that *Sonic hedgehog* expression is also maintained by the AER and can be rescued by FGF-4. In fact, *Bmp-2* expression might be a direct response of cells to secreted Sonic hedgehog protein. *Bmp-2* itself is a candidate for a secondary signaling molecule in the cascade of patterning events induced by *Sonic hedgehog*. *Bmp-2* is a secreted molecule of the transforming growth factor β family, and its expression can be induced by *Sonic hedgehog*.

This appears to be an evolutionarily conserved pathway, as *hedgehog* (the Drosophila homolog of *Sonic hedgehog*) activates the expression of *decapentaplegic* (the homolog of *Bmp-2*) in the eye and wing imaginal discs (Heberlein et al., 1993; Ma et al., 1993; Tabata and Kornberg, 1994). Expression of *hedgehog* is normally confined to the posterior of the wing disc. Ectopic expression of *hedgehog* in the anterior of the disc results in ectopic expression of *decapentaplegic* and, ultimately, in the duplication of wing structure with mirror-image symmetry (Basler and Struhl, 1994; Fietz et al., 1995). This effect is strikingly parallel to the phenotypic results of ectopic expression of *Sonic hedgehog* in the chick limb.

Application of BMP-2 protein does not, however, induce digit pattern alterations (Francis et al., 1994). It is possible that *Bmp-2* is only able to effect changes in pattern in concert with other signals downstream of *Sonic hedgehog*, or perhaps with *Sonic hedgehog* itself.

Regulation of *Sonic hedgehog* Expression

Sonic hedgehog expression is activated in the posterior of the limb bud very early during mesodermal outgrowth (Riddle et al., 1993). The factors that initiate this localized expression are not yet identified, but ectopic expression of *Hoxb8* at the anterior margin of the mouse limb bud results in the activation of a second domain of *Sonic hedgehog* expression under the anterior AER (Charité et al., 1994). Since retinoic acid is known to be able to induce the expression of *Hoxb-8* and other *Hox* genes in vitro (Mavilio et al., 1988), it is possible that endogenous retinoic acid acts to make cells competent to express *Sonic hedgehog* by inducing expression of upstream *Hox* genes, either in the very early limb bud or in the flank prior to limb bud formation.

Once induced, *Sonic hedgehog* expression is dependent on signals from the posterior AER. Following its initiation in the posterior limb mesoderm, the *Sonic hedgehog* expression domain moves distally as the limb bud grows out, always remaining subjacent to the AER. Similarly, *Sonic hedgehog* expression can also be induced on the anterior margin of the limb bud by implantation of a retinoic acid bead, but the induced ectopic expression is limited to the mesoderm directly underlying the AER (Riddle et al., 1993). In addition, ZPA activity fades rapidly following removal of the AER (Niswander et al., 1993; Vogel and Tickle, 1993), and ZPA grafts only function when placed in close proximity to the AER (Tabin, 1991; Tickle, 1991). Our observation that continued *Sonic hedgehog* expression depends on signals from the posterior AER reveals a mechanism underlying these observations.

The reliance of *Sonic hedgehog* expression on AER-derived signals suggests an explanation for the distal shift in *Sonic hedgehog* expression during limb development (Riddle et al., 1993). Signals from the AER also promote distal outgrowth of the mesodermal cells of the progress zone, which in turn results in the distal displacement of the AER. Hence, as maintenance of *Sonic hedgehog* expression requires signals from the AER, its expression domain will be similarly displaced.

We find that replacement of the AER with FGF-4-soaked beads results in the maintenance of *Sonic hedgehog* ex-

pression. This result is consistent with the previous findings that ZPA activity can be maintained in ovo and in vitro by members of the FGF family (Anderson et al., 1993; Niswander et al., 1993; Vogel and Tickle, 1993). Since *Fgf-4* is normally expressed in the posterior AER, these results suggest that *Fgf-4* is the signal from the ectoderm involved in maintaining *Sonic hedgehog* expression.

Sonic hedgehog and Regulation and Maintenance of the AER

Sonic hedgehog can induce anterior extensions of the AER that have an inverted polarity relative to the endogenous AER. This polarity is demonstrated by examining the expression of two markers in the AER. In normal limbs, *Bmp-2* is expressed throughout the AER, while *Fgf-4* is expressed in the posterior two-thirds of the AER. In the extended AER resulting from ectopic Sonic hedgehog expression, *Bmp-2* is again found throughout the AER, while *Fgf-4* expression is biphasic, found at either end of the AER, and overlies the anterior and posterior mesodermal domains expressing *Sonic hedgehog*. These results are consistent with previous observations that anteroposterior polarity of the AER appears to be regulated by the underlying mesoderm and that ZPA grafts lead to the induction of ectopic, polarized AER tissue (Maccabe and Parker, 1979). Our results also suggest that the normal AP polarity of the AER is a reflection of endogenous *Sonic hedgehog* expression. The induced AER is sufficient to promote complete PD outgrowth of the induced structures (Riddle et al., 1993). Hence, whatever factors are necessary to maintain the AER are also downstream of *Sonic hedgehog*.

A Positive Feedback Loop between *Sonic hedgehog* and *Fgf-4*

The induction of *Fgf-4* expression by *Sonic hedgehog* in the ectopic AER and the maintenance of *Sonic hedgehog* expression by FGF-4 suggest that *Sonic hedgehog* and *Fgf-4* expression are normally sustained by a positive feedback loop. Such a feedback loop allows the coordination of mesodermal outgrowth and patterning. This coordination is possible because *Sonic hedgehog* patterns mesodermal tissue and regulates *Fgf-4* expression, while FGF-4 protein induces mesodermal proliferation and maintains *Sonic hedgehog* expression. Moreover, mesodermal tissue can only be patterned by *Sonic hedgehog* in the context of a competence activity provided by FGF-4. Thus, patterning is always coincident with proliferation.

It remains possible that exogenously applied FGF-4 might be mimicking the activity of a different member of the FGF family. For example, FGF-2 is expressed in the limb mesoderm and the AER (Dono and Zeller, 1994; Savage et al., 1993) and has similar effects on limb tissue as does FGF-4 (Niswander and Martin, 1993; Niswander et al., 1993; Riley et al., 1993; Fallon et al., 1994).

Coordinated Regulation of Limb Outgrowth and Patterning

Our results suggest that *Sonic hedgehog* acts as a short range signal that triggers a cascade of secondary signals the interplay of which determines the resultant pattern of

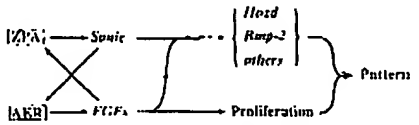

Figure 7. Model for the Coordinated Growth and Patterning of the Limb

Sonic hedgehog is proposed to signal directly to the mesoderm to induce expression of the *Hoxd* and *Bmp-2* genes. The induction of these mesodermal genes requires competence signals from the overlying AER. One such signal is apparently *Fgf-4*. Expression of *Fgf-4* in the AER can be induced by *Sonic hedgehog*, providing an indirect signaling pathway from *Sonic hedgehog* to the mesoderm. FGFs also maintain expression of *Sonic hedgehog* in the ZPA, thereby completing a positive feedback loop that controls the relative positions of the signaling centers. While *Fgf-4* provides competence signals to the mesoderm, it also promotes mesodermal proliferation. Thus, patterning of the mesoderm is dependent on the same signals that promote its proliferation. This mechanism inextricably integrates limb patterning with outgrowth.

structures. The data suggest a number of inductive pathways that can be combined to generate a model (Figure 7) that describes how *Sonic hedgehog*, in coordination with the AER, acts to pattern mesodermal tissues along the AP limb axis while simultaneously regulating PD outgrowth.

Following its induction, *Sonic hedgehog* signals to both the limb ectoderm and mesoderm. *Sonic hedgehog* imposes a distinct polarity on the forming AER, including the posteriorly biased expression of *Fgf-4*, and the AER becomes dependent on continued *Sonic hedgehog* expression. The mesoderm, as long as it is receiving permissive signals from the overlying ectoderm, responds to the *Sonic hedgehog* signal by expressing secondary signaling molecules such as *Bmp-2* and by activating *Hoxd* genes. *Bmp-2* expression is directly dependent on continued *Sonic hedgehog* expression, while the continued expression of the *Hoxd* genes rapidly becomes *Sonic hedgehog* independent. In a reciprocal fashion, maintenance of *Sonic hedgehog* expression in the posterior mesoderm becomes dependent on signals from the AER. Since the factors expressed by the AER are not only required for the maintenance of *Sonic hedgehog* expression and activity but are also mitogenic, growth and patterning become inextricably linked. Coordination of limb development through interdependent signaling centers forces the AP and PD structures to be induced and patterned in tandem. The pathways elucidated in this study thus provide a molecular framework for the controls governing limb patterning.

Experimental Procedures

Unless otherwise noted, all standard cloning techniques were performed according to Ausubel et al. (1989), and all enzymes and molecular biology reagents were obtained from Boehringer Mannheim Biochemicals. Sequences were analyzed using both Genetics Computer Group (Devereux et al., 1984) and DNAstar software (Madison, Wisconsin). Searches for related sequences were done through the BLAST network service (Altschul et al., 1990) provided by the National Center for Biotechnology Information.

Cloning of Chicken *Fgf-4* and *Bmp-2*

A 246 bp fragment of the chicken *Fgf-4* gene was cloned by PCR

from a stage 22 chicken limb bud library. Degenerate primers were designed against previously cloned *Fgf-4* and *Fgf-8* genes: fgf5' (sense), 5'-AAA AGC TTT AYT GYT AYG TIG GIA THG G-3'; and fgf3' (antisense), 3'-AAG AAT TCT AIG CRT TRT ART TRT TIG G-5'. Denaturation was at 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 50°C for 60 sec, and 72°C for 30 s, with a final extension at 72°C for 5 min. The PCR product was subcloned into the Bluescript SK(+) vector. A clone was sequenced and confirmed as *Fgf-4* by comparison to previously published *Fgf-4* genes and a chicken *Fgf-4* gene sequence provided by L. Niswander.

BMP-related sequences were amplified from a stage 22 posterior limb bud cDNA library prepared in Bluescript using primers and conditions as described by Basler et al. (1993). Amplified DNAs were cloned and used to screen a stage 22 limb bud library prepared in λZAP (Stratagene). Among the cDNAs isolated was chicken *Bmp-2*. Its identity was confirmed by sequence comparison to the published clones (Francis et al., 1994) and by its expression patterns in chick embryos.

Chick Surgeries and Recombinant Retroviruses

All experimental manipulations were performed on White Leghorn chick embryos (standard specific pathogen–free, S-SPF) provided by SPAFAS (Norwich, Connecticut). Eggs were staged according to Hamburger and Hamilton (1951).

Viral supernatants of *Sonic hedgehog*/RCAS-A2 or a variant containing an influenza hemaglutinin epitope tag at the carboxyl terminus of the *Sonic hedgehog* protein (*Sonic hedgehog*7.1/RCAS-A2, functionally indistinguishable from *Sonic hedgehog*/RCAS-A2) were prepared as described (Hughes et al., 1987; Fekete and Cepko, 1993; Riddle et al., 1993). For focal injections, the right wings of stage 18–21 embryos were transiently stained with nile blue sulfate (0.01 mg/ml in Ringer's solution) to reveal the AER. A trace amount of concentrated viral supernatant was injected beneath the AER.

The AER was removed using electrolytically sharpened tungsten wire needles. Some embryos had a heparin–acrylic bead soaked in FGF-4 solution (0.8 mg/ml; a gift from the Genetics Institute) or PBS stapled to the limb bud with a piece of 0.025 mm platinum wire (Goodfellow [Cambridge, England]) essentially as described by Niswander et al. (1993).

Limbs that were infected with *Sonic hedgehog*/RCAS virus after AER removal were infected over a large portion of the denuded mesoderm to ensure substantial infection. Those embryos that received both an FGF-4–soaked bead and virus were infected only underneath the bead.

In Situ Hybridizations and Photography

Single-color whole-mount in situ hybridizations were performed as described (Riddle et al., 1993). Two-color whole-mount in situ hybridizations were performed essentially as described by Jowett and Lettice (1994). The second color detection was developed using 0.125 mg/ml magenta-phos (Biosynth International [Skokie, Illinois]) as the substrate. Radioactive in situ hybridizations on 5 μm sections were performed essentially as described by Tassarollo et al. (1992).

The following probes were used for whole-mount and section in situ hybridizations: *Sonic hedgehog*, a 1.7 kb fragment of pHH2 (Riddle et al., 1993); *Bmp-2*, a 1.5 kb fragment encoding the entire open reading frame; *Fgf-4*, a 250 bp fragment described above; *Hoxd-11*, a 600 bp fragment; *Hoxd-13*, a 400 bp fragment both including 5' untranslated sequences and coding sequences upstream of the homeobox; and RCAS, a 900 bp Sall–ClaI fragment of RCAS (Hughes et al., 1987).

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